Graphical Abstract

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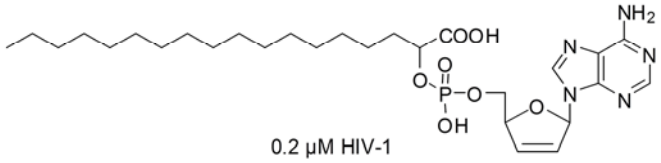

Hydroxy Fatty Acids For The Delivery Of Dideoxynucleosides As Anti-HIV Agents

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ABSTRACT

A series of α- and β-carboxylated phospholipid prodrugs of dideoxy nucleosides have been synthesized and evaluated against HIV. An increase in biological effect with a factor of 500 has only been observed for the adenine nucleoside, which suggests that this prodrug approach is base specific.

Keywords:
Prodrugs
Nucleotides
Phospholipids
d4A
HIV

2',3'-Dideoxynucleosides and 2',3'-didehydro-2',3'-dideoxynucleosides are nucleoside reverse transcriptase inhibitors (NRTIs). Some of them have been found effective for the treatment of HIV infections. These NRTIs[1] inhibit the viral replication by terminating DNA polymerization. One of the efforts to improve the therapeutic potential of nucleoside analogues for treating viral infections is directed towards prodrug development.

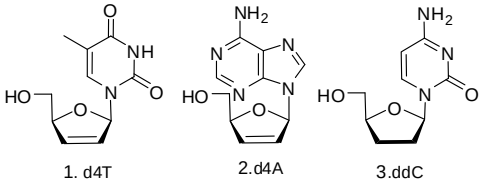
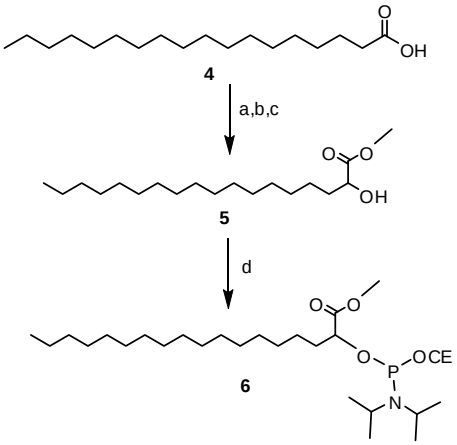


Figure 1. Structure of anti-HIV nucleosides

Prodrug approaches have been developed for increasing oral absorption and cellular penetration of modified nucleos(t)ides. In addition, prodrugs are also used for tissue specific delivery of nucleotides, and for kinase bypass strategies. These prodrug strategies have the aim to mask the anionic phosphate moiety of nucleotides, and to deliver the modified nucleos(t)ides (in blood or in cells) involving enzymatic degradation reactions. One of the approaches that was followed in the past is synthesis of phospholipid prodrugs of modified nucleosides. Hostetler et al has demonstrated that the acyclovir diphosphate dimeristoylglycerol prodrug delivers acyclovir monophosphate inside cells.[2] Acyl nucleotide[3] derivatives of d4T and AZT, as well as O-alkyl-5',5'-dinucleoside phosphates of AZT/cordycepin[4] have been synthesized. Generally, the anti-HIV efficacy of the prodrug was lower[3,4] or similar[3] than that of the parent nucleotide. Many more phosphodiester prodrugs of modified nucleosides have been studied[5] but generally it is believed that mono alkyl/aryl phosphodiesters are unsuitable for the intracellular delivery of nucleotides.[6] In the case of d4A and d4C, 5'-methyl and 5''-phenyl phosphate diesters shows in vitro anti-HIV activity comparable to those of the parent nucleoside.[7] In the present work we report the synthesis and antiviral activity of phospholipid conjugates of d4A, d4T, ddC. Two types of lipids have been considered i.e. (±)-α-hydroxy stearic acid and (±)-β-hydroxy stearic acid. It is hypothesized that the lipid moiety may help cell penetration while the α-carboxylic acid function may assist in phosphodiester bond cleavage.[8] Dependent on which ester bond is cleaved (C5'-O-P or CH-O-P), either the nucleoside or the nucleotide can be delivered in the cell. Additional factors which may complicate the predictability of the results are that the stability of 5'-phosphodiester conjugates of nucleoside with an α-carboxylate group could be base dependent and that the role of phosphodiesterase enzymes in the cleavage of the lipid conjugates is unknown. Intramolecular catalysis of P-O or P-N bond cleavage (to liberate the nucleoside or nucleotide by chemical degradation of the prodrug) occurs via a 5-membered (α-COOH) or a 6-membered (β-COOH) intermediate, and both possibilities have been investigated. A prodrug of AZT was not included in this study. To evaluate the potential of a new prodrug

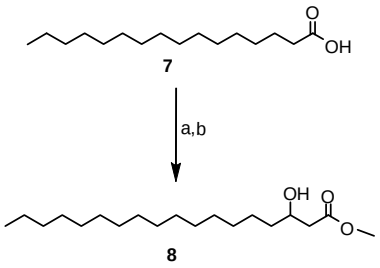
concept, results are more pronounced when using a lower active or almost inactive nucleoside as model compound, rather than a very potent congener.

Stavudine (d4T) **1** is prepared by a two-step method starting from thymidine as previously reported.[9] The synthesis of 2', 3'-didehydro-2', 3'-dideoxyadenosine **2** has been accomplished as previously described,[10,11] by converting the vicinal diol of adenosine to olefin using Corey-Winter reaction conditions. The nucleoside ddC **3** is commercially available. The methyl (±)-α-hydroxy stearate **5** is prepared in three steps from stearic acid **4** by bromination[12] using Hell-Volhard-Zelinsky conditions followed by hydrolysis[13] and esterification[14] in 88 % overall yield (Scheme 1).



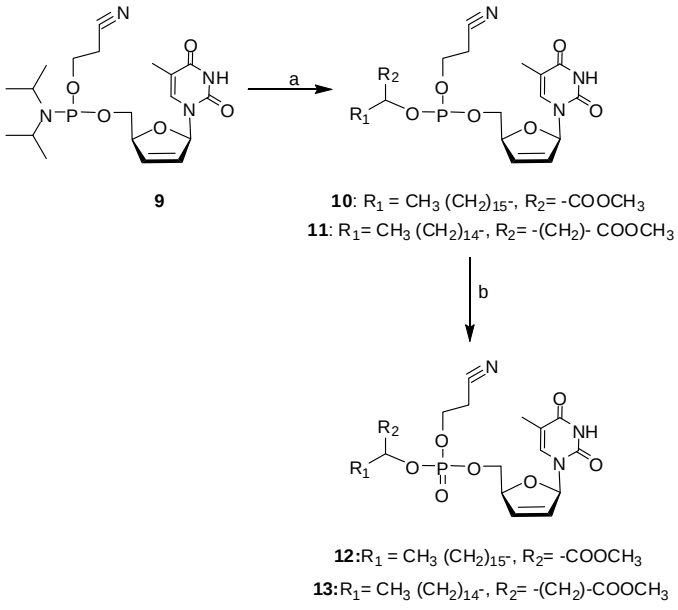
Scheme 1. a) PBr_3, Br_2, 95°C, 6h; b) aq.NaOH, 85°C, 3h; c) TMSCl, DMP, MeOH, r.t. overnight; d) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, dry DCM, 1H-tetrazole, 0°C-r.t. 15min.

The methyl (±)-β-hydroxy stearate **8** was synthesized by the procedure described by Masamune[15] which involves homologation of palmitic acid **7** using in situ generated magnesium monomethylmalonate[16] to a preformed acyl imidazole to produce the β-keto stearic acid methyl ester, which is reduced[17] with sodium borohydride in ethanol providing **8**[18] in 77 % overall yield (Scheme 2).

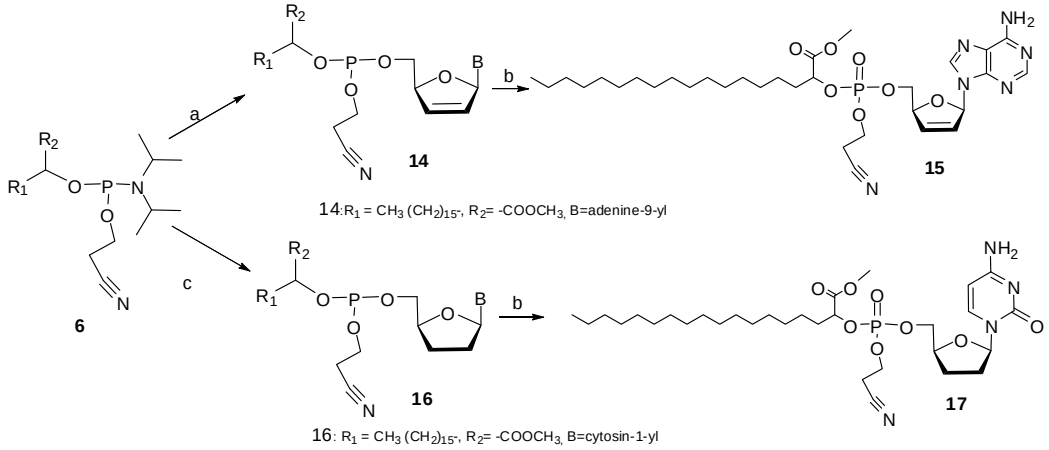


Scheme 2. a) CDI, $MgCl_2$, $K^+ {}^-O_2CCH_2CO_2CH_3$, THF, r.t. overnight; b) $NaBH_4$, EtOH, 0°C-r.t. 15 min.

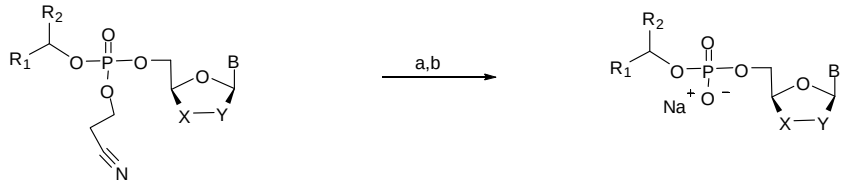
The phosphoramidite approach was used for the synthesis of the lipid-nucleotide conjugates. In the case of thymine nucleoside, the 5'-O-phosphoramidite of d4T (**9**)[19] was coupled with the hydroxylated fatty acid esters **5** and **8** yielding the phosphite intermediates **10** and **11** which were subsequently oxidized using aqueous iodine in Pyridine/THF resulting in the formation of **12** and **13**. (Scheme 3).



Scheme 3. a) **5** for **10** and **8** for **11**, 1H-tetrazole, dry DCM, r.t. 4h; b) aq. iodine in pyridine/THF, r.t. 30 min



Scheme 4. a) **2**,1H-tetrazole, dry DCM, r.t. 4h; b) aq. iodine in pyridine/THF, r.t. 30 min; c) **3**, 1H-tetrazole, dry DCM, r.t. 4h.



12: $R_1 = CH_3(CH_2)_{15}$-, R_2 = -$COOCH_3$, X-Y = CH=CH, B=thymin-1-yl
13: $R_1 = CH_3(CH_2)_{14}$-, R_2 = -(CH_2)-$COOCH_3$, X-Y = CH=CH, B=thymin-1-yl
15: $R_1 = CH_3(CH_2)_{15}$-, R_2 = -$COOCH_3$, X-Y = CH=CH, B=adenin-9-yl
17: $R_1 = CH_3(CH_2)_{15}$-, R_2 = -$COOCH_3$, X-Y = CH_2-CH_2, B=cytocin-1-yl

18: $R_1 = CH_3(CH_2)_{15}$-, R_2 = -$COONa^+$, X-Y = CH=CH, B=thymin-1-yl
19: $R_1 = CH_3(CH_2)_{14}$-, R_2 = -(CH_2)-$COONa^+$, X-Y = CH=CH, B=thym-1-yl
20: $R_1 = CH_3(CH_2)_{15}$-, R_2 = -$COONa^+$, X-Y = CH=CH, B=adenine-9-yl
21: $R_1 = CH_3(CH_2)_{15}$-, R_2 = -$COONa^+$, X-Y = CH_2-CH_2; B=cytosin-1-yl

Scheme 5. a) aq. Ammonia:MeOH (1:0.5) , r.t. 36h b) Ion exchange, Na+ form.

In the case of cytosine and adenine nucleotides, the phosphoramidite derivative of the (±)-α-hydroxy stearic methyl ester **6** was first synthesized and coupled with the nucleoside analogue. The phosphoramidite **6** was obtained as a diastereomeric mixture in 56% yield. The reverse approach (as used for the synthesis of the thymine analogues) is lower yielding.

The d4A and ddC conjugates were obtained by the reaction of **6** with d4A and ddC, followed by oxidation of the intermediates **14** and **16** to produce **15** and **17** respectively (Scheme 4). Deprotection of the phosphotriesters **12**, **13**, **15** and **17** was first carried out by removal of the cyanoethyl protecting group with DBU in THF, followed by hydrolysis of the methyl ester with 1N sodium hydroxide in MeOH. However, the yields were moderate and several side products were formed. Therefore we turned to a one-step deprotection procedure using an aqueous ammonia/MeOH mixture. (Scheme 5).

The lipid-nucleotide conjugates **18-21** were purified on a Dowex-50 Na^+ column followed by lyophilization. Biological activity was determined on the diastereomeric mixtures.

The activity of the phosphodiester analogues of d4A-**20**, ddC-**21**, d4T-**18**, **19** against HIV-1 and HIV-2 was determined including the dideoxy nucleosides d4A, ddC, d4T as reference compounds. The anti-HIV activity and cytotoxicity of the compounds were evaluated against wild-type HIV-1 and HIV-2 in MT-4 cell cultures. Anti-HIV-2 activity was also determined in CEM cells as well as in thymidine kinase-deficient CEM (CEM/TK-) cell cultures. The α-hydroxy D4T derivative **18** has an IC50 of 0.4 μM against HIV-1 and HIV-2 in MT-4 cells, but was not active in CEM cells. Compound **19** (the β–hydroxy d4T conjugate) is about 30 times less active than the α–hydroxy d4T analogue. However both compound **18** and **19** are less active than the parent nucleoside (d4T). Similar results are obtained for the ddC lipid conjugate **21** i.e. the parent nucleoside (ddC) is more active than the potential prodrug **21**, as well in MT-4 cells as in CEM/0 cells.

Between the three α-hydroxy nucleoside conjugates tested, d4A takes a particular place. The parent d4A itself is an anti-HIV nucleoside with very low activity as well in MT-4 cells as in CEM/0 cells. Conjugation of d4A-MP with α-hydroxy stearic acid leads to an increase in activity of at least a factor 500 in MT-4 cells. The activity is similar against HIV-1 and against HIV-2. Previously, a methyl phosphate diester was reported as a potential prodrug of d4A,[7] however, with no increase in antiviral potency. The observation that the activity of **20** in CEM/K- cells is lower than in CEM/0 cells suggests that the prodrug of d4A does not deliver d4A-MP in the cells.

In conclusion, hydroxy fatty acid conjugates of d4T-MP, d4A-MP and ddC-MP have been prepared as prodrugs for their potential ability to deliver the antiviral nucleosides and/or nucleotides into the cell. Only in the case of d4A, increased antiviral activity against HIV-1 and HIV-2 has been observed. It therefore seems that this prodrug approach is base specific. The inactivity of the d4A conjugate in CEM/TK^- cells suggests that the modified nucleosides rather than the nucleotide is delivered in the infected cells. We previously observed that the chemical stability of α-carboxylated phosphoramidates of nucleosides is base dependent.[8] Some initial stability studies have been performed on the dA and dT analogues of compounds **18**, **19** and **20**. All compounds are stable in neutral and basic (pH12) medium. Degradation of the α-COOH compounds occurs in acidic circumstances, liberating dA or dT and the phosphorylated lipid within a few days at pH=6 and within minutes at pH=4. These results suggest that chemical degradation might not be the reason for delivering d4A inside cells, but this is not conclusive. The study in acidic circumstances has been performed in DMSO (as all compounds from gels in aqueous acetic medium) which questions the (biological) relevance of this experiment. It could therefore be possible that **20** is more easily enzymatically degraded in cells than **18**, **19** or **21**.

Acknowledgments

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/***. Here the experimental section is also included.

Table 1. Antiviral activity test of compounds against HIV-1 and HIV-2

	MT-4 cells [a]			CEM/0 [b]		CEM/TK$^-$ [c]
Analogue	IC_{50} (μM) HIV-1	IC_{50} (μM) HIV-2	CC_{50} (μM)	IC_{50} (μM) HIV-2	CC_{50} (μM)	IC_{50} (μM) HIV-2
18	0.4±0.1	0.4±0.1	64.7±8.7	>39.6	92.6±9.5	>39.6
19	10.9±1.0	16.2±0.2	>198.2	>198.2	>198.2	>39.6
20	0.19±0.02	0.11±0.01	97.8±5.5	25.3 ± 12.7	81.3±8.7	>39.1
21	9.7±2.5	5.7±0.1	94.1±8.8	7.5 ± 2.5	91.1±8.3	>40.5
d4T	0.26±0.03	0.4±0.1	327.4±32.2	8.8 ± 6.4	>267.6	≥ 119.7
d4A	>277.5	>277.5	277.5±19.2	⩾36.6	360.3±63.7	⩾81.9
ddC	1.0±0.6	1.9±0.3	324.8±29.4	0.5±0.3	178.8±33.0	1.9±0.8
AZT	0.007±0.002	0.007±0.002	>93.5	0.04±0.01	>467.7	>14.9

a HIV-1 (IIIB), HIV-2 (ROD); b CEM/0 cells (ROD); c CEM thymidine kinase deficient cells (ROD)

References and notes

1. Martin, J. C.; Hitchcock, M. J.; De Clercq, E.; Prusoff, W. H., *Antiviral Res.* **2010**, *85*, 34.
2. Hostetler, K. Y.; Parker, S.; Sridhar, C. N.; Martin, M. J.; Li, J. L.; Stuhmiller, L. M.; van Wijk, G. M.; van den Bosch, H.; Gardner, M. F.; Aldern, K. A., *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11835
3. Bonnaffé, D., Dupraz, B., Ughetto-Monfrin, J., Namane, A., Henin, Y., Huynh Dinh, T., *J. Org. Chem.* **1996**, *61*, 895
4. Meier, C., Neumann, J.M., Andre, F., Henin, Y., Huynh Dinh, T., *J. Org. Chem.* **1992**, *57*, 7300
5. Wagner, C. R.; Iyer, V. V.; McIntee, E. J., *Med. Res. Rev.* **2000**, *20*, 417.
6. Krise, J. P.; Stella, V. J., *Adv. Drug Deliv. Rev.* **1996**, *19*, 287.
7. Mullah, K. B.; Rao, T. S.; Balzarini, J.; De Clercq, E.; Bentrude, W. G., *Journal of Medicinal Chemistry* **1992**, *35*, 2728.
8. Maiti, M.; Michielssens, S.; Dyubankova, N.; Maiti, M.; Lescrinier, E.; Ceulemans, A.; Herdewijn, P., *Chem. Eur. J.* **2012**, *18*, 857.
9. Horwitz, J. R.; Chua, J.; Da Rooge, M. A.; Noel, M.; Klundt, I. L., *J. Org. Chem.* **1966**, *31*, 205.
10. Dudycz, L. W., *Nucleos. Nucleot.* **1989**, *8*, 35.
11. Chu, C. K.; Bhadti, V. S.; Doboszewski, B.; Gu, Z. P.; Kosugi, Y.; Pullaiah, K. C.; Vanroey, P., *J. Org. Chem.* **1989**, *54*, 2217.
12. Polyakova, S. M.; Belov, V. N.; Yan, S. F.; Eggeling, C.; Ringemann, C.; Schwarzmann, G.; de Meijere, A.; Hell, S. W., *Eur. J. Org. Chem.* **2009**, 5162.
13. Masuda, Y.; Mori, K., *Eur. J. Org. Chem.* **2005**, 478.
14. Horneff, T.; Herdtweck, E.; Randoll, S.; Bach, T., *Bioorg. Med. Chem.* **2006**, *14*, 6223-6234.
15. Brooks, D. W.; Lu, L. D. L.; Masamune, S., Angew. Chem. Int. Ed. Eng. **1979**, *18*, 72.
16. Ratovelomanana-Vidal, V.; Girard, C.; Touati, R.; Tranchier, J. P.; Hassine, B. B.; Genêt, J. P., *Adv. Syn. Catal.* **2003**, *345*, 261.
17. Yang, D.; Gao, Q.; Zheng, B.-F.; Zhu, N.-Y., *J. Org. Chem.* **2004**, *69*, 8821.
18. Bergström, S.; Aulin-Erdtman, G.; Rolander, B.; Stenhagen, E.; Östling, S., *Acta Chem Scand.* **1952**, *6*, 1157.
19. Abraham, T. W.; Wagner, C. R., *Nucleos. Nucleot.* **1994**, *13*, 1891.